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## 54 Recombinant Humicola lipase and process for the production of recombinant humicola lipases.

5) A process for the production of a recombinant Humicola lipase is provided. The process comprises cultivating an *Aspergillus* sp. host transformed with a vector system comprising DNA sequences encoding functions facilitating gene expression and optionally a suitable marker for selection of transformants, and a DNA sequence encoding the Humicola lipase. A preferred host organism is *Aspergillus oryzae*. The recombinant Humicola lipase from *A. oryzae* differs from the native lipase in having a greater glycosylation and in exhibiting an improved thermostability.

4	14	24	34	44	54
Aliphatic alcohols					
44	74	94	94	104	124
Aliphatic aldehydes					
124	134				
Carboxylic acids					

## Description

## RECOMBINANT HUMICOLA LIPASE AND PROCESS FOR THE PRODUCTION OF RECOMBINANT HUMICOLA LIPASES

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## BACKGROUND OF THE INVENTION

10 The present invention relates to a process for recombinant DNA production of Humicola lipases and a recombinant Humicola lipase.

15 Humicola lipases are obtainable from strains of thermophilic Humicola sp., including thermophilic Thermomyces sp., such as H. lanuginosa (Griffon and Maublanc) Bunce, H. stellata Bunce, H. grisea var. thermoidea, Cooney & Emerson, H. insolens, Cooney & Emerson, Thermomyces ibadanensis, Apinis & Eggin, H. hyalothermophila Moubasher, Mazen and Abdel-Hafez, H. grisea var. indica Subrahmanyam, H. brevis var. thermoidea, Subrahmanyam and Thirumalachar and H. brevispora Subrahmanyam and Thirumalachar.

20 H. lanuginosa has also been described under the synonyms Thermomyces lanuginosus Tsiklinsky, Sepedonium lanuginosum Griffon and Maublanc, Sepedonium thermophilum cyclosporum and S. thermophilum ovoспорум Velich, Acremoniella sp. Rege, Acremoniella thermophila Curzi and Monosporopsis lanuginosa (Griffon and Maublanc) Mason.

25 Moreover, the species Scytalidium thermophilum (Cooney & Emerson) Austwich was by Hedger (1975, The ecology of thermophilic fungi in Indonesia. In Biodegradation et Humification. Rapport du 1<sup>er</sup> Colloque International - Nancy 1974 (ed. G. Kilbertius, O. Reisinger, A. Mourey & J.A. Cancela Da Fonseca), Sarreguemines: Pierron Editeur - 57206) considered to belong to Humicola insolens.

30 Production of a Humicola lanuginosa lipase is described in Japanese unexamined patent publication No. 48-62990, and in EP patent application No. 87307684.8. The latter also teaches use of this lipase in lipolytic detergent additives.

35 Due to the world wide use of enzyme additives in detergents and due to the fact that Humicola lipases have turned out to be superior to known detergent lipases both as regards detergency and stability, commercial interest in such lipases is high.

40 In the production of industrial enzymes yields have always been important for the profitability of the production process. The traditional way of improvement is to mutate the wild strain so as to obtain higher yielding mutants. By means of recombinant DNA technology a further possibility is to transform the gene for the desired product into a host microorganism capable of producing higher yields than the wild strain or with other favourable characteristics.

45 Accordingly, it is the aim of the present invention to develop a method for the production of Humicola lipases by recombinant DNA-technology.

50 In the past, numerous processes have been developed for the production of polypeptides or proteins by means of the recombinant DNA technology. The main interest has been concentrated on bacteria and yeast, e.g. E. coli, Bacillus subtilis and Saccharomyces cerevisiae being well characterized species as regards for instance expression and selection systems.

55 Besides the above mentioned microorganisms, filamentous fungi, notably Aspergillus sp. such as Aspergillus niger and Aspergillus oryzae, being well-characterized and widely used microorganisms for the commercial production of enzymes, are attractive candidates as host microorganisms for recombinant DNA vectors.

60 In the last few years different selection markers for the transformation of Aspergillus nidulans have been described and procedures have recently been developed for integrative transformation of the filamentous fungus Aspergillus nidulans for the purpose of investigation of the genetic and molecular processes controlling fungal cell differentiation.

65 Transformation of A. nidulans has been demonstrated by using plasmids containing the Neurospora crassa pyr-4 gene (Ballance, D.J. et al., Biochem.Biophys.Res.Commun., 112 (1983) 284-289), the A. nidulans amdS gene (Tilburn, J.G. et al., Gene 26 (1983) 205-221), the A. nidulans trpC gene (Yelton, M.M. et al., Proc.Natl.Acad.Sci. U.S.A., 81 (1984) 1470-1474) and the A. nidulans argB gene (John, M.A. and Peberdy J., Microb.Technol. 6 (1984) 386-389). The transforming DNA was found to be integrated into the host genome at rather low frequencies (typically < 1000 transformants/ $\mu$ g of DNA).

70 Transformation of Aspergillus niger with the amdS gene of A. nidulans was described (Kelly, J.M. and Hynes, M.J., EMBO Journal 4 (1985), 475-479) and amdS was shown to be a potential selection marker for use in transformation of Aspergillus niger that cannot grow strongly on acetamide as a sole nitrogen source. Transformation of Aspergillus niger using the argB gene of Aspergillus nidulans has also been described (Buxton, F. P. et al., Gene 37 (1985), 207-214).

75 A process for preparing transformants of Aspergillus niger is described in EP No. 0184,438A and EP No. 0215,594 and 0249,350 describe expression of heterologous polypeptides in filamentous fungi. Finally WO87/04464 describes expression of higher eucaryotic genes in Aspergillus.

## BRIEF DESCRIPTION OF THE INVENTION

According to the present invention it has now been shown that it is possible to obtain a high level of expression of the Humicola sp. lipase in Aspergillus sp. strains or to enhance the production of the lipase in Humicola strains.

In its broadest aspect the present invention provides a method for the production of Humicola lipases comprising the steps of

- (a) providing a suitable recombinant DNA cloning vector comprising DNA sequences encoding functions facilitating gene expression and a DNA sequence encoding the Humicola lipase;
- (b) transforming a suitable host organism with the cloning vector from step (a);
- (c) culturing the transformed host in a suitable culture medium and optionally recovering the lipase from the culture medium.

In a more narrow aspect of the present invention there is provided a process for the production of Humicola lipases in Aspergillus comprising the steps of:

(a) providing a recombinant DNA cloning vector system capable of integration into the genome of an Aspergillus host in one or more copies and comprising: DNA sequences encoding functions facilitating gene expression; a suitable marker for selection of transformants; and a DNA sequence encoding the Humicola lipase;

(b) transforming the Aspergillus host which does not harbour a functional gene for the chosen selection marker with the recombinant DNA cloning vector system from step a; and

(c) culturing the transformed Aspergillus host in a suitable culture medium and optionally recovering of the lipase from the culture medium.

According to a second aspect of the present invention there is provided a method for production of Humicola lipases in Aspergillus by which method an Aspergillus strain being transformed with a recombinant DNA cloning vector system as described above is cultured in a suitable culture medium and the lipase is recovered from the culture medium.

According to a third aspect of the invention there is provided a novel recombinant Humicola lipase product characterized by a difference in glycosylation from the native Humicola lipases previously known. That is, the nature and optionally the extent of glycosylation of the recombinant Humicola lipase of the present invention is different from the lipase obtained from the naturally occurring Humicola strains. The Humicola lipase product of this invention is furthermore characterized by an improved thermostability compared to the corresponding native Humicola lipase.

More specifically the novel recombinant Humicola lipase is characterized in that the carbohydrate content is of about the same level or is greater than the carbohydrate content in the native lipase whereas the nature of the glycosylation is different from the native Humicola lipase, i.e. the novel Humicola lipase contains other carbohydrates than the native lipase. The carbohydrate content may typically be from about 5 to about 30% (w/w), whereas the carbohydrate content in the native lipase is about 4.9% (w/w). More specifically the carbohydrate content may be in the range from about 5 to about 15% (w/w) and even more specifically it may be in the range from about 7.5% to about 8.5% (w/w).

The recombinant Humicola lipase product of this invention may be used as an enzymatic detergent additive for use in detergents in a similar way as the native lipase, i.e. as described in EP patent application No. 87307684.8.

As used in the present specification the term "recombinant Humicola lipase" is applied to Humicola lipase produced by culturing a microorganism transformed with the cDNA encoding the native Humicola lipase. The term "native Humicola lipase" is applied to the Humicola lipase obtained from natural sources of thermophilic Humicola sp., including thermophilic Thermomyces sp. described in the introductory part of the present specification.

It has been found that recombinant Humicola lipase from A. oryzae is not identical with native Humicola lipase, notwithstanding that the peptide sequence is the same. Apparently the host microorganism glycosylates the expressed polypeptide to a different extent than the donor microorganism and with different sugar moieties. In A. niger the extent of glycosylation seems to be on the same level whereas the sugar moieties seem to be of the same kind as in A. oryzae. Differentiation and identification of the native lipase and of the recombinant lipase is possible through measurement of their glycosylation. Thus, taking as exemplary the native Humicola lanuginosa lipase and the recombinant form of this lipase from an A. oryzae transformant, both lipases are N-glycosylated, but with different sugar residues. The native lipase does not have any galactose and has less mannose than the recombinant lipase and also is glycosylated to a different degree, the native lipase having carbohydrate moieties that add approximately 1500 Daltons to its molecular weight (about 5%) and the recombinant lipase having moieties that add about 2600 Daltons (about 8%). For details see Example 5 hereinafter.

The novel recombinant Humicola lipase according to the present invention may comprise from about 1 to about 12 mol galactose per mol lipase protein and more specifically from about 1 to about 6 mole galactose per mol lipase protein. The content of mannose may be from about 3 to about 20 and more specifically from

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about 3 to about 12 mol mannose per mol lipase protein. The recombinant Humicola lipase will typically comprise about 2 mol N-acetylglucosamine, from about 3 to about 20 mol mannose and from about 1 to about 12 mol galactose per mol lipase protein. More specifically the recombinant Humicola lipase will contain about 2 mol N-acetylglucosamine, about 3 to about 12 mol mannose and about 1 to about 6 mol galactose per mol lipase product. Even more specifically the recombinant Humicola lipase will contain about 2 mol N-acetylglucosamine, about 6 to about 9 mol mannose and about 2 to about 4 mol galactose per mol lipase protein.

5 The differences in glycosylation have some effect on the enzyme properties. In particular, the thermal stability of highly purified H. lanuginosa recombinant lipase is superior vis a vis the thermal stability of comparably purified native lipase. In addition, the stability of the pure recombinant lipase in the presence of an alkaline Bacillus protease (Esperase<sup>®</sup>) tested at 40°C and at 55°C is superior vis a vis comparably pure native lipase.

10 Characteristically, enzyme products, here lipase products, particularly extracellular lipase products contain other enzyme activities notably proteolytic activity and non-enzymatically active peptides and amino acids derived from culture broth constituents.

15 In the instance of the Humicola sp. lipase, the recombinant lipase product has been found to be thermally more stable than the comparable native lipase product. According to the data of the inventors hereof part of the improvement in thermal stability may be attributed to the different glycosylation, and another part may be attributed to the absence of the Humicola proteolytic activity in the recombinant lipase product. In the instance 20 of the lipase native to H. lanuginosa a brief description thereof in U.S. patent 4,707,291 reports that a commercially available H. lanuginosa lipase product (Amano CE) contained a substantial level of proteolytic activity. The inventors' native H. lanuginosa lipase product contained a comparable level of proteolytic activity. At elevated temperatures in particular the protease can be expected to degrade other enzymes. The improvement in thermal stability attributable to absence of the protease is cumulative of the improvement 25 attributable to different glycosylation (see Example 6).

20 As has already been pointed out the present invention provides a method for producing high yields of the Humicola lipases by cultivation of transformed Aspergillus strains, and in a preferred embodiment a method for production of the Humicola lanuginosa lipase by cultivation of a transformant A. oryzae or A. niger host cell. A. oryzae is the most preferred host microorganism.

30 A. oryzae has for years been used in commercial scale for the production of the TAKA-amylase enzyme and of proteolytic enzymes, and accordingly fermentation technology for cultivating this microorganism is well developed and the microorganism itself is approved for use in the food industry. The present invention offers the possibility of using A. oryzae for industrial production of large quantities of recombinant Humicola lipases.

35 The present invention furthermore provides a recombinant Humicola lipase product superior to the native Humicola lipase for having increased thermostability, and increased stability in the presence of alkaline Bacillus proteases, see Figures 6 and 9 herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

40 The present invention is further illustrated by reference to the accompanying drawings in which:  
Fig. 1 shows the DNA-sequence of the TAKA-amylase promoter and upstream promoter regions, the preregion and the 5'part of the structural gene for the TAKA-amylase.  
Fig. 2 shows an endonuclease restriction map of plasmid pTAKA17.  
Fig. 3 illustrates the construction of plasmid pHL.  
45 Fig. 4 illustrates the construction of plasmid p960.  
Fig. 5a and b shows the DNA sequence of prepro Humicola lanuginosa lipase cDNA together with the deduced amino acid sequence given by three-letter abbreviations.  
Fig. 6 shows the residual activity of the recombinant Humicola lipase product compared to the residual activity of the native Humicola lipase product at 55°C and 60°C at different pH.  
50 Fig. 7 shows an SDS-PAGE gradient gel.  
Fig. 8 shows the residual activity at 55°C of the recombinant Humicola lipase product and the native Humicola lipase product along with the residual activity of the native Humicola lipase containing a protease inhibitor (PMSF), and  
Fig. 9 shows the residual activity at 40°C and at 55°C of native and recombinant Humicola lipase in the 55 presence of an alkaline Bacillus protease product (Esperase<sup>®</sup>)

#### DETAILED DESCRIPTION OF THE INVENTION

60 The transformation technique used for the transformation of Aspergillus strains was a method adapted from the methods for transformation of A. nidulans (Ballance et al. Biochem.Biophys.Res.Commun., 112 (1983), 284-289; Tilburn et al., Gene 26 (1983), 205-221, Yelton et al. Proc.Natl.Acad.Sci. USA, 81 (1984), 1470-1474) and similar to the method of Buxton et al. (Gene 37 (1985), 207-214) for transformation of A. niger. In the process of the present invention an Aspergillus strain is transformed with a vector system containing a selection marker which is capable of being incorporated into the genome of the host strain, but which is not harboured in the host strain before the transformation. Transformants can then be selected and isolated from 65

nontransformants on the basis of the incorporated selection marker.

Preferred selection markers are the argB (A. nidulans or A. niger), trpC (A. nidulans), amdS (A. nidulans), or pyr4 (Neurospora crassa) genes, or the DHFR (dihydrofolate reductase or mutants hereof) gene. More preferred selection markers are the argB or the amdS gene. Wild type A. oryzae strains are normally argB<sup>+</sup> (i.e. the argB gene is functional in A. oryzae). If argB is chosen as the selection marker an argB mutant strain of A. oryzae which has a defect in the gene for this marker must be used as host strain. A. oryzae argB mutants can be prepared as described by F.P. Buxton et al. (Gene 37 (1985), 207-214). An argB mutant is defined as a mutant having a defect in the ornithine transcarbamylase gene. On the other hand the amdS gene may be used as selection marker for the transformation of wild type A. oryzae as the wild type strains do not contain this gene.

DNA-sequences encoding functions facilitating gene expression are typically promoters, transcription terminators and polyadenylation signals.

The promoter, which might be preceded by upstream activating sequences and enhancer sequences as well known in the art, may be any DNA-sequence that might show strong transcriptional activity in Aspergillus and may be derived from genes encoding both extracellular and intracellular proteins, such as amylases, glucoamylases, proteases, lipases, cellulases and glycolytic enzymes. Suitable promoters may be derived from genes for A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger glucoamylase, A. niger neutral  $\alpha$ -amylase, A. niger acid stable  $\alpha$ -amylase, and Rhizomucor miehei lipase. Examples of promoters from genes for glycolytic enzymes are TPI, ADH and PGK.

A preferred promoter according to the present invention is the A. oryzae TAKA-amylase promoter. The TAKA amylase is a well known  $\alpha$ -amylase (Toda et al., Proc.Japan Acad. 58 Ser. B (1982) 208-212). DNA encoding the promoter region was derived from the TAKA-amylase genomic clone as described in EP patent application No. 87103806.3. The sequence of the promoter and regions upstream to the promoter together with the preregion and the 5'end of the structural gene for the TAKA-amylase is illustrated in Fig. 1.

The TAKA-amylase promoter is available from plasmid pTAKA 17 being deposited in connection with EP patent application No. 87103806.3. The endonuclease restriction map of plasmid pTAKA17 is shown in fig. 2.

From pTAKA 17 the whole promoter sequence including sequences upstream to the promoter or functional parts thereof may be derived by means evident to the person skilled in the art. The promoter sequence may be provided with linkers with the purpose of introducing specific restriction sites facilitating the ligation of the promoter sequence with further DNA, for instance the gene encoding the desired protein product or different preregions (signal peptides).

In the method according to the present invention the sequence from nucleotide -1144 (see Fig. 1) (representing a Sall site) to nucleotide -10 has been used as one example of a well functioning part of the promoter region. In another embodiment of the present invention the nucleotide sequence from nucleotide -1176 to -1 was preceded by the still not sequenced 1.05 kb fragment from pTAKA 17. It is evident for the person skilled in the art that different fragments can be used.

According to one embodiment of the present invention the promoter and upstream activating sequences have the following sequence or a functionally equivalent nucleotide sequence:

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GTCGACGC ATTCCGAATA CGAGGCCTGA TTAATGATTA CATA CGCCTC  
CGGGTAGTAG ACCGAGCAGC CGAGCCAGTT CAGCGCCTAA AACGCCTTAT  
5 ACAATTAAGC AGTTAAAGAA GTTAAATCT ACGCTTAAAAGCTACTTAA  
AAATCGATCT CGCAGTCCCG ATTGCCTAT CAAAACAGT TTAAATCAAC  
TGATTAAGG TGCCGAACGA GCTATAATG ATATAACAAT ATTAAAGCAT  
10 TAATTAGAGC AATATCAGGC CGCGCACGAA AGGCAACTTA AAAAGCGAAA  
GCGCTCTACT AAACAGATTA CTTTGAAAA AGGCACATCA GTATTTAAAG  
CCCGAATCCT TATTAAGCGC CGAAATCAGG CAGATAAAAGC CATA CAGGCA  
15 GATAGACCTC TACCTATTAA ATCGGCTTCT AGGCGCGCTC CATCTAAATG  
TTCTGGCTGT GGTGTACAGG GGCATAAAAT TACGCACTAC CCGAATCGAT  
AGAACTACTC ATTTTTATAT AGAAAGTCAGA ATTCA TAGTG TTTTGATCAT  
20 TTTAAATTT TATATGGCGG GTGGTGGCA ACTCGCTTGC GC GGGCAACT  
CGCTTACCGA TTACGTTAGG GCTGATATTT ACGT GAAAAT CGTCAAGGGA  
TGCAAGACCA AAGTAGTAAA ACCCCGGAAG TCAACAGCAT CCAAGCCAA  
25 GTCCTTCACG GAGAAACCCC AGCGTCCACA TCACGAGCGA AGGACCACCT  
CTAGGCATCG GACGCACCAT CCAATTAGAA GCAGCAAAGC GAAACAGCCC  
AAGAAAAAGG TCGGCCCGTC GGCCTTTCT GCAACGCTGA TCACGGCAG  
30 CGATCCAACC AACACCCCTCC AGAGT GACTA GGGGCGGAAA TTTAAAGGGA  
TTAATTTCCA CTCAACCAACA AATCACAGTC GTCCCCGGTA TTGTCTGCA  
GAATGCAATT TAAACTCTTC TGCGAATCGC TTGGATTCCC CGCCCTAGT  
35 CGTAGAGCTT AAAGTATGTC CCTTGTCGAT GCGATGTATC ACAACATATA  
AATACTAGCA AGGGATGCCA TGCTTGGAGG ATAGCAACCG ACAACATCAC  
ATCAAGCTCT CCCTTCTCTG AACAAATAAAC CCCACAG representing the  
40 sequence from nucleotide -1144 to -10 in Fig. 1.

45 According to a further embodiment the promoter and upstream activating sequences have the following sequence or a functionally equivalent nucleotide sequence:

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AGATCTGCCCTTATAAATCT CCTAGTCTGA TCGTCGACGC ATTCCGAATA  
 CGAGGCCTGA TTAATGATTA CATA CGCCTC CGGGTAGTAG ACCGAGCAGC  
 CGAGCCAGTT CAGGCCCTAA AACGCCCTAT ACAATTAAGC AGTTAAAGAA 5  
 GTTAGAATCT ACGCTAAAAA AGCTACTTAA AAATCGATCT CGCAGTCCCG  
 ATT CGCCTAT CAAAACCAGT TTAAATCAAC TGATTAAGG TGCCGAACGA  
 GCTATAAATG ATATAACAAT ATTAAAGCAT TAATTAGAGC AATATCAGGC 10  
 CGCGCACGAA AGGCAACTTA AAAAGCGAAA GCGCTCTACT AAACAGATTA  
 CTTTGAAAAA AGGCACATCA GTATTTAAAG CCCGAATCCT TATTAAGCGC  
 CGAAATCAGG CAGATAAAGC CATA CAGGCA GATAGACCTC TACCTATTA 15  
 ATCGGCTTCT AGGCGCGCTC CATCTAAATG TTCTGGCTGT GGTGTACAGG  
 GGCATAAAAT TACGCACTAC CCGAATCGAT AGAACTACTC ATTTTTATAT  
 AGAAGTCAGA ATT CATAGTG TTTTGATCAT TTTAAATTT TATATGGCGG 20  
 GTGGTGGGCA ACTCGCTTGC CGGGGCAACT CGCTTACCGA TTACGTTAGG  
 GCTGATATT ACGTGAAAAT CGTCAAGGGGA TGCAAGACCA AAGTAGTAAA  
 ACCCCGGAAG TCAACAGCAT CCAAGCCCAA GTCCTTCACG GAGAAACCCC 25  
 AGCGTCCACA TCACGAGCGA AGGACCACCT CTAGGCATCG GACGCACCAT  
 CCAATTAGAA GCAGCAAAGC GAAACAGCCC AAGAAAAAGG TCGGCCCGTC  
 GGCCTTTCT GCAACGCTGA TCACGGGAG CGATCCAACC AACACCCCTCC 30  
 AGAGTGACTA GGGGCGGAAA TTTAAAGGGGA TTAATTTCCA CTCAACCACA  
 AATCACAGTC GTCCCCGGTA TTGTCCTGCA GAATGCAATT TAAACTCTTC  
 TCGGAATCGC TTGGATTCCC CGCCCTAGT CGTAGAGCTT AAAGTATGTC 35  
 CCTTGTGAT GCGATGTATC ACAACATATA AATACTAGCA AGGGATGCCA  
 TGCTTGGAGG ATAGCAACCG ACAACATCAC ATCAAGCTCT CCCTTCTCTG  
 AACAAATAAAC CCCACAGAAG GCATTT representing the sequence from 40  
 nucleotide -1176 to -1 in Fig. 1. 45

According to a further aspect of the present invention the latter sequence may be preceded by the 1.05 kb unsequenced upstream region from pTAKA 17 (position 0 to 1.05 in Fig. 2).

The terminators and polyadenylation sequences may be derived from the same sources as the promoters. Enhancer sequences may also be inserted into the construction.

The expressed product may be accumulated within the cells requiring disruption of the cells to isolate the product. To avoid this further process step and also to minimize the amount of possible degradation of the expressed product within the cells it is preferred that the product is secreted from the cells. For this purpose the gene for the desired product is provided with a preregion ensuring effective direction of the expressed product into the secretory pathway of the cell. This preregion which might be a naturally occurring signal or leader peptide or functional parts thereof or a synthetic sequence providing secretion is generally cleaved from the desired product during secretion leaving the mature product ready for isolation from the culture broth.

The preregion may be derived from genes for secreted proteins from any source of organism.

According to the present invention the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the  $\alpha$ -factor from *S. cerevisiae* or a *Humicola* lipase gene. More preferably the preregion may be derived from the gene for the *Humicola lanuginosa* lipase, *A. oryzae* TAKA amylase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid-stable  $\alpha$ -amylase, *B. licheniformis*  $\alpha$ -amylase, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus*  $\alpha$ -amylase or *B. licheniformis* subtilisin. The *Humicola lanuginosa* signal peptide has the following sequence

ATGAGGAGGTCCCTTGTGCTGTTCTCTGCGTGGACGGCCTGGCC  
MetArgSerSerLeuValLeuPhePheValSerAlaTrpThrAlaLeuAla

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The TAKA-amylase signal has the following sequence

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ATGATGGTCGCGTGGTGGTCTCTATTCTGTACGGCCTTCAGGTGCGGGCACCT  
MetMetValAlaTrpTrpSerLeuPheLeuTyrGlyLeuGlnValAlaAlaPro

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GCTTTGGCT  
AlaLeuAla

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The gene for the desired product functionally linked to promoter and terminator sequences may be incorporated in a vector containing the selection marker or may be placed on a separate vector or plasmid capable of being integrated into the genome of the host strain. As used herein the expression "vector system" includes a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA-information to be integrated into the host genome. Vectors or plasmids may be linear or closed circular molecules. According to a preferred embodiment of the present invention the host organism is cotransformed with two vectors, one including the selection marker and the other comprising the remaining foreign DNA to be introduced in the host strain, including promoter, the gene or cDNA for the desired product and transcription terminator and polyadenylation sequences.

Normally the transformants are stable and can be cultured in the absence of a selection pressure. If the transformants turn out to be unstable the selection marker may be used for selection during cultivation. The transformed cells are then cultured under a selection pressure corresponding to the marker in question.

35 Plasmids used as starting materials in the following examples are as follows:

p285: (ATCC No. 20681)  
pSal43: Berse et al. Gene 25 (1983), 109-117 John & Peberdy, Enzyme Microb. Technol. 6 (1984), 386-389.  
p3SR2: J.M. Kelly and M.J. Hynes, EMBO Journal 4 (1985), 475-479.  
pSP62-K2 and pCDVI-PL: Noma et al. Nature, 319, (1986), 640-646)  
40 p775: European patent application No. 87103806.3,  
pUC19: Vieira et al., Gene 19 (1982), 259- 268 and Messing, Meth. in Enzymology 101 (1983), 20-27.

The strains used are as follows:

*A. oryzae*\*: ATCC 20423, IFO 4177, ATCC 1011, ATCC 9576, ATCC 14488-11491, ATCC 11601 and ATCC 12892.

45 *E. coli*: MC1000 (Casabandan, M.J. and Cohen, S.N., J.Mol.Biol. 138, 179-207) (NCIB 11956)

*H. lanuginosa*: DSM 4109

50 *A. niger*\*: ATCC 1015, ATCC 10582.

\* ArgB mutants of these strains can be prepared as described by F.P., Buxton et al. (Gene 37 (1987) 207-214). An ArgB mutant is defined as a mutant having a defect in the ornithine transcarbamylase gene.

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#### Identification of *Humicola lanuginosa* lipase (HLL) amino acid sequence

In order to obtain information which allows the construction of a specific oligonucleotide probe, a partial sequence determination was carried out on the purified *Humicola lanuginosa* lipase. The supernatant from a culture broth of *Humicola lanuginosa*, from which mycelia and low molecular weight substances had been removed was subjected to a column chromatography performed by use of DEAE-sepharose (anion exchange chromatography), phenyl sepharose (hydrophobic interaction chromatography) followed by gel filtration on TSK G3000 SW. The sequence determination was performed with a Gas Phase Sequencer (Applied Biosystems Model 470A) as described by Thim, L. et al. (FEBS Lett. 212, 307-312 (1987)).

60 The following N-terminal sequence was found:

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This sequence allows the construction of two specific mixed oligonucleotide probes comprising the sequences from amino acid residue No. 7 - No. 11 (Phe-Asn-Gln-Phe-Asn) and amino acid residue No. 13 - No. 16 (Phe-Ala-Gln-Tyr), respectively. The screening of a *Humicola lanuginosa* cDNA library by use of HLL-specific oligonucleotide mixtures corresponding to the above sequences is described in example 1.

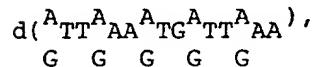
### Example 1

## Construction of a *Humicola lanuginosa* cDNA library in *E. coli*

Total RNA was extracted from homogenized *H. lanuginosa* mycelium using methods as described by Boel et al. (EMBO J., 3: 1097-1102, 1984) and Chirgwin et al., (Biochemistry (Wash.), 18, 5294-5299, 1979). Poly(A)-containing RNA was obtained by two cycles of affinity chromatography on oligo(dT)-cellulose as described by Aviv and Leder (PNAS, USA, 69, 1408-1412, 1972). cDNA was synthesized with the use of methods described by Okayama and Berg (Molec.Cell.Biol., 2: 161-170, 1982), and with the vectors pSP62-K2 and pCDVI-PL described by Noma et al. (Nature, 319: 640-646, 1986). The synthesized cDNA was transformed into a *hsdR*<sup>-</sup>, *M<sup>r</sup>* derivative of *E. coli* MC1000 (Casadaban and Cohen, J. Mol.Biol., 138, 179-207, 1980) to generate recombinant clones.

### Identification of *Humicola lanuginosa* lipase (HLL) specific cDNA recombinants

### A mixture of 32 pentadecamer oligodeoxyribonucleotides



one of which is complementary to HLL mRNA in the region coding for Phe-Asn-Gln-Phe-Asn was synthesized on an Applied Biosystems, Inc. DNA synthesizer and purified by polyacrylamide gel electrophoresis. Approximately 10,000 *E. coli* recombinants from the *Humicola lanuginosa* cDNA library were transferred to Whatman 540 paper filters. The colonies were lysed and immobilized as described by Gergen et al. (Nucleic Acids Res. 7, 2115-2135, 1979). The filters were hybridized with the  $^{32}$ P-labelled HLL-specific pentadecamer-mixture as described by Boel et al. (EMBO J. 3, 1097-1102, 1984). Hybridization and washing of the filters were done at 37°C and 43°C respectively, followed by autoradiography for 24 hours with an intensifier screen. Miniprep plasmid DNA was isolated from two hybridizing colonies, pHLL 702.3 and pHLL 702.4 by standard procedures (Birnboim and Doly, Nucleic Acids Res., 7, 1513-1523, 1979), and the DNA sequence of the cDNA insert was established by the procedure of Maxam and Gilbert (Methods Enzymol. 65, 499-560, 1980).

to facilitate further construction work with the HLL cDNA, DNA sequences containing unique restriction sites were added to the 5' and the 3' ends of the cDNA as follows. pHLL 702,3 was digested with Sau96I which digests HLL cDNA in the 3' untranslated region and the resulting ends were filled in with *E. coli* DNA polymerase (Klenow fragment) and the four dNTPs. This DNA was subsequently digested with SacI which cuts the HLL cDNA once just 3' to the initiating methionine codon. The resulting 0.9 kb HLL cDNA fragment was purified by agarose gel electrophoresis, electroeluted and made ready for ligation reactions. As a 5' adaptor two oligonucleotides, 927 and 928, were synthesized. The sequence of the adaptor is shown in fig. 3. This adaptor was designed to add a HindIII and a BamHI site just 5' to the initiating Met codon of HLL cDNA. The two oligos were kinased with ATP and T4 polynucleotide kinase, annealed to each other and ligated to the purified 0.9 kb HLL cDNA sequence in a pUC19 vector that had been digested with HindIII and HinclI and purified on a 0.7% agarose gel. The resulting plasmid pHLL carried the HLL cDNA as a portable 0.9 kb BamHI fragment. The construction of pHLL is shown in fig. 3.

After BamHI digestion and purification of the 0.9 kb HLL cDNA fragment on an agarose gel it was ligated to BamHI digested and phosphatased p775 to generate p960 in which HLL cDNA is under transcriptional control of the TAKA promotor from Aspergillus oryzae and the AMG terminator from Aspergillus niger. The construction of p960 is shown in fig. 4.

The construction of p775 is described in European patent application No. 87103806.3.

p775 contains the TAKA promoter and AMG terminator and has a unique BamHI site as a cloning site. Figure 5a and b gives the sequence of prepro HLL cDNA with its deduced amino acid sequence. Nucleotides are numbered from the first base in the cloned cDNA. From this cDNA sequence it can be concluded that HLL is synthesized as a 291 amino acid residue long precursor with a signal peptide of 17 residues, and a short propeptide of 5 residues. The putative signal peptidase processing site (von Heijne, Eur.J.Biochem. 133, 17-21, 1983) is indicated with an arrow pointing to the peptide bond between an Ala and a Ser residue. The amino terminus of the mature enzyme as identified by amino terminal amino acid sequencing is indicated.

10 Amino acid composition of *Humicola lanuginosa* (HLL)

15 Amino acid analysis was carried out by means of a Beckman Amino Acid Analyzer (Model JL1 MB) on samples (40 µg) previously hydrolyzed in sealed ampoules in 6 M HCl or 4 M methanesulfonic acid at 110°C for 24, 48 and 96 hours. Half-cystine was determined as S-β-(4-pyridylethyl)-cysteine after reduction by tributylphosphine followed by coupling with 4-vinylpyridine. All chemicals were of highest purity.

20 The results are shown in the following table and compared to the amino acid composition determined from cDNA sequencing.

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	Amino Acid	Found	Nearest integer	cDNA
	Ala	20.85	21	21
25	Arg	14.42	14	14
	Asn	36.91	37	19
	Asp			19
30	Cys <sup>c</sup>	5.54	6	6
	Gln	18.20	18	6
	Glu			12
35	Gly	27.51	28	28
	His	5.94	6	6
	Ile <sup>b</sup>	14.99	15	16
40	Leu	20.06	20	20
	Lys	7.05	7	7
	Met	0	0	0
45	Phe	14.80	15	15
	Pro	11.90	12	12
	Ser <sup>a</sup>	16.76	17	17
50	Thr <sup>a</sup>	18.46	18	19
	Trp	3.58	4	4
	Tyr	9.84	10	10
55	Val <sup>b</sup>	18.24	18	18

60 a) extrapolated value to zero hydrolysis time

b) extrapolated value to infinite hydrolysis time

c) determined as S-β-(4-pyridylethyl)-cysteine.

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Example 2Transformation of Aspergillus oryzae or Aspergillus niger (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) was inoculated with spores of *A. oryzae*, *A. niger* or *argB* mutants hereof and incubated with shaking at 37°C for about 2 days. The mycelium was harvested by filtration through miracloth and washed with 200 ml of 0.6 M MgSO<sub>4</sub>. The mycelium was suspended in 15 ml of 1.2 M MgSO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH = 5.8. The suspension was cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234, batch 1687 was added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) was added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts was visible in a sample inspected under the microscope.

The suspension was filtered through miracloth, the filtrate transferred to a sterile tube and overlaid with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation was performed for 15 min. at 1000 g and the protoplasts were collected from the top of the MgSO<sub>4</sub> cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl pH = 7.5, 10 mM CaCl<sub>2</sub>) were added to the protoplast suspension and the mixture was centrifuged for 5 min. at 1000 g. The protoplast pellet was resuspended in 3 ml of STC and repelleted. This was repeated. Finally the protoplasts were resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension was mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts from the *argB* strains were mixed with pSal43 DNA (an *A. nidulans* *argB* gene carrying plasmid) and protoplasts from the *argB*<sup>+</sup> strains were mixed with p3SR2 (an *A. nidulans* *amdS* gene carrying plasmid). The mixture was left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl<sub>2</sub> and 10 mM Tris-HCl pH = 7.5 was added and carefully mixed (twice) and finally 0.85 ml of the same solution was added and carefully mixed. The mixture was left at room temperature for 25 min., spun at 2500 g for 15 min. and the pellet was resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts were spread on the appropriate plates. Protoplasts from the *argB* strains transformed with pSal43 were spread on minimal plates (Cove, Biochem.Biophys.Acta 113 (1966) 51-56) with glucose and urea as carbon and nitrogen sources respectively, and containing 1.2 M sorbitol for osmotic stabilization. Protoplasts from the *argB*<sup>+</sup> strains transformed with p3SR2 were spread on minimal plates (Cove, Biochem.Biophys.Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores were picked, suspended in sterile water and spread for single colonies. this procedure was repeated and spores of a single colony after the second reisolation were stored as a defined transformant.

Example 3Expression of recombinant Humicola lipase (RHL) in an *A. oryzae* strain

p960 is transformed into *A. oryzae* IFO 4177 by cotransformation with p3SR2 containing the *amdS* gene from *A. nidulans* as described with a mixture of equal amounts of p960 and p3SR2 (approximately 5 µg of each). Transformants which can use acetamide as sole nitrogen source are reisolated twice. After growth on YPD (Sherman et al, 1981) for three days culture supernatants are analysed by SDS-PAGE. The gels are stained with coomassie brilliant blue R. The best transformants are selected for further studies and grown in a 2 liter Kiefer fermentor on 4% soy bean meal and supplied with glucose during growth. The culture is heavily agitated during fermentation. The recombinant *Humicola* lipase product (RHL) was isolated from the culture broth by removal of the cells by centrifugation, ultrafiltration of the supernatant and freeze drying.

Example 4Expression of Humicola lipase in an *A. niger* strain

p960 was transformed into *A. niger* *argB* by cotransformation with pSal43 containing the *argB* gene from *A. nidulans* described in Example 2. Protoplasts were incubated with equal amounts, approximately 5 µg, of each plasmid. Transformants were selected on minimal plates (Cove Biochim. Biophys. Acta 113 (1966), 55-56) by relief of arginine requirement.

After two reisolations of conidiospores the transformants were cultured for seven days in YPD (Sherman et al., 1981) at 30°C. The culture supernatants were analyzed by SDS-PAGE. Most of the transformants produced *Humicola* lipase in their supernatants.

The carbohydrate content was analyzed by Endo H treatment as described in Example 5. It was found that the carbohydrate content was of about the same magnitude as for the native *Humicola* lipase. The Endo H sensitivity was the same as for the recombinant *Humicola* lipase from *A. oryzae*. It was accordingly assumed

that the nature of the glycosylation is the same in A. oryzae and A. niger.

Example 5

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Determination of carbohydrate content in RHL (recombinant lipase product from example 3) and HLL (native lipase product from cultivation of DSM4109).

10 Treatment with enzymes capable of cleaving off carbohydrate side chains (Endo H and Glycopeptidase F).

RHL and HLL were treated with glycopeptidase F (Boehringer No. 91378, 100 units. 1 vial dissolved in 500 µ water) and Endo H (Sigma No. E6878, dissolved in citrate buffer pH 5.5).

15 HLL and RHL were each dissolved in 10 mM Tris pH 7.5 (1 mg/ml). To 100 µl HLL and RHL, respectively 2 µl glycopeptidase F was added and the samples were incubated at 37°C for 20 hours.

HLL and RHL were furthermore each dissolved in 10 mM Tris pH 7.5 (1 mg/ml) and 25 µl Endo H and 50 µl 0.1 M sodium acetate pH 5.0 were added to 25 µl of HLL and RHL, respectively. The samples were incubated at 37°C for 20 hours.

20 The samples were run on SDS-PAGE gradient gels 7.5-20% together with untreated HLL and RHL. The SDS-PAGE gradient gel is shown in fig. 7. The samples in fig. 7 are as follows:

- 1) Standard: 92K, 67K, 43K, 30K, 20.1K, 14.4K
- 2) HLL, 1 mg/ml in 10 mM Tris pH 7.5.
- 3) HLL + Endo H
- 4) RHL + Endo H
- 5) RHL + Glycopeptidase F
- 6) HLL + Glycopeptidase F
- 7) RHL + Endo H
- 8) RHL + Glycopeptidase F
- 9) RHL, 1 mg/ml in 10 mM Tris pH 7.5.

30 It appears from fig. 7 that glycopeptidase F is capable of cleaving off the carbohydrate part of both HLL and RHL and that the remaining protein is of the same size. Endo H is on the other hand only capable of cleaving off carbohydrates of RHL whereas Endo H treatment of HLL does not have any effect.

Accordingly, both HLL and RHL are N-glycosylated. The glycosylation is, however, of a different nature. Carbohydrate analysis was performed by use of methods described by Thim et. al., submitted for publication in Biochemistry, Chaplin, M.F. (1982), Anal.Biochem. 123, 336-341, and Jentoft, N. (1985), Anal.Biochem. 148, 424-433.

40 Briefly, samples containing lipase was subjected to methanolysis followed by re-N-acetylation of the amino sugars and elimination of O-acetyl groups by a second, mild methanolysis step. Derivatives suitable for analysis by reverse phase high pressure liquid chromatography was obtained by perbenzoylation of the methyl glycosides.

The result of the analysis is shown in the following table.

45	Native	Recombinant
	<u>Humicola lipase</u>	<u>Humicola lipase</u>
	<u>HLL</u>	<u>RHL</u>
	<u>mol/mol</u>	<u>mol/mol</u>
50	N-acetylglucosamine	1.2
	Mannose	8.6
55	Galactose	3.3

60 The carbohydrate moiety of the native Humicola lipase, HLL is composed of the two monosaccharides found in N-glycosylations of the high-mannose type (Montrenil, J. et al., (1986) in "Carbohydrate analysis: a practical approach", Chaplin, M.F. and Kenndey, J.F. (Eds.), IRL Press, Oxford, p 143). The result for N-acetylglucosamine (< 2 mol/mol) indicates that the primary sequence only contains a single N-glycosylation. This could further be deduced from the cDNA sequence (Fig. 5a + 5b). In addition mannose could be O-glycosidic linked to serine or threonine residues.

65 Besides N-acetylglucosamine and mannose, the recombinant Humicola lipase, RHL comprises galactose in significant amount. As for the native lipase the content of N-acetylglucosamine indicates the presence of a

single N-glycosylation, although this is of the complex or hybrid type if galactose is part hereof. O-glycosylation at serine or threonine residues, however, has to be responsible for the presence of galactose if the N-glycosylation of the recombinant RHL lipase is of the high-mannose type as was the case for the native HLL lipase.

In average, the carbohydrate moieties add approximately 1500 D and 2600 D to the molecular weight of the native Humicola lipase HLL and the recombinant Humicola lipase RHL, respectively. This corresponds to a carbohydrate content of about 4.9% and 8.1%, respectively.

#### Example 6

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##### Effect of pH and temperature on the stability of RHL and HLL

RHL ( $1.4 \times 10^6$  LU/g) and HLL ( $0.2 \times 10^6$  LU/g), respectively, were dissolved in buffer solutions of various pH and incubated for two hours at temperatures 55° and 60°C.

The buffer solutions were made up of 47.5 mM Naacetate, MOPS (3-(N-morpholino)-propane sulphonic acid), and boric acid, with pH-adjustments to 4, 5, 6, 7, 8, 9, and 10 by means of 1 N HCl or 1 N NaOH. In addition the buffer solution used in a separate test of a sample of HLL that had been purified to  $1.5 \times 10^6$  LU/g was added PMSF (phenylmethan sulfonyl fluorid) to inhibit protease content in this native lipase product.

Lipase concentrations in the solutions were equated to approx. 10-15 LU/ml.

Immediately after the incubations the lipase solutions were cooled down in an ice-water bath and kept there until analysis the same day (LU-method, AF 95).

The results are given in fig. 6 and in fig. 8.

It appears from fig. 6 that the thermostability of RHL is greater than that of HLL at pH 5-10 both at 55°C and 60°C, respectively.

It appears from fig. 8 that the greater thermostability of RHL is due only in part to absence of the H. lanuginose protease activity.

#### Example 7

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#### Claims

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Stability of RHL and HLL of comparable unit activity levels of  $4 \times 10^6$  LU/g in the presence of a typical alkaline Bacillus sp. protease, (here the commercially available detergent enzyme Esperase®).

The incubation conditions were 0.1 M boric acid, pH 9.5 and 40°C or 55°C using concentrations of 3600 LU/liter and 0.057 AU/liter (AU/LU =  $0.016 \times 10^{-3}$ ). The residual activities were calculated on the basis of reference incubations.

The results are given in Figure 9.

It appears from Fig. 9 that the thermostability of RHL in presence of an alkaline Bacillus protease is superior to that of HLL, indicating thereby differences in their susceptibility to attack by proteases.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

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1. A process for the production of a recombinant Humicola lipase comprising the steps of
  - (a) providing a suitable recombinant DNA cloning vector comprising DNA-sequences encoding functions facilitating gene expression and a DNA-sequence encoding the Humicola lipase;
  - (b) transforming a suitable host organism with the cloning vector from step (a);
  - (c) culturing the transformed host in a suitable culture medium and optionally recovering the lipase from the culture medium.
2. A process according to claim 1, wherein the host is an Aspergillus strain and comprising the steps of:
  - (a) providing a recombinant DNA cloning vector system capable of integration into the genome of a suitable Aspergillus host in one or more copies and comprising: DNA sequences encoding functions facilitating gene expression; a suitable marker for selection of transformants; and a DNA sequence encoding the Humicola lipase;
  - (b) transforming the Aspergillus host which does not harbour a functional gene for the chosen selection marker with the recombinant DNA cloning vector system from step a; and
  - (c) culturing the transformed Aspergillus host in a suitable culture medium and optionally recovering the lipase from the culture medium.
3. A process according to claim 2, wherein the DNA-sequences encoding functions facilitating gene expression comprises a promoter, transcription initiation sites, and transcription terminator and polyadenylation functions.

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4. A process according to claim 3, wherein the promoter is preceded by upstream activating sequences.
5. A process according to claim 2, wherein the selection marker is derived from the gene for A. nidulans or A. niger argB, A. nidulans trpC, A. nidulans amdS, Neurospora crassae Pyr4 or DHFR.
6. A process according to claim 5, wherein the selection marker is the ArgB gene derived from A. nidulans or A. niger or the amdS gene derived from A. nidulans.
7. A process according to claim 4, wherein the promoter and upstream activating sequences are derived from a gene encoding an extracellular or intracellular protein, such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.
8. A process according to claim 7, wherein the promoter and upstream activating sequences are derived from the gene for A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral  $\alpha$ -amylase, A. niger acid stable  $\alpha$ -amylase, A. niger glucoamylase or Rhizomucor miehei lipase.
9. A process according to claim 2, wherein the Aspergillus host is an Aspergillus oryzae strain.
10. A process according to claim 9, wherein the promoter is the A. oryzae TAKA amylase promoter or functional parts thereof.
15. 11. A process according to claim 10, wherein the promoter and upstream activating sequences have the following sequence:

20 GTCGACGC ATTCCGAATA CGAGGCCTGA TTAATGATTA CATA CGCCTC  
 CGGGTAGTAG ACCGAGCAGC CGAGCCAGTT CAGCGCCTAA AACGCC TTAT  
 ACAATTAAGC AGTTAAAGAA GTTAAATCT ACGCTTAAAA AGCTACTTAA  

25 AAATCGATCT CGCAGTCCCG ATTGCGCTAT CAAAACCAGT TTAAATCAAC  
 TGATTAAGG TGCCGAACGA GCTATAATG ATATAACAAAT ATTAAAGCAT  
 TAATTAGAGC AATATCAGGC CGCGCACGAA AGGCAACTTA AAAAGCGAAA  

30 GCGCTCTACT AAACAGATTA CTTTGAAAA AGGCACATCA GTATTTAAAG  
 CCCGAATCCT TATTAAGCGC CGAAATCAGG CAGATAAAGC CATA CAGGCA  
 GATAGACCTC TACCTATTAA ATCGGCTTCT AGGCGCGCTC CATCTAAATG  

35 TTCTGGCTGT GGTGTACAGG GGCATAAAAT TACGCACTAC CCGAATCGAT  
 AGAACTACTC ATTTTTATAT AGAAGTCAGA ATTCA TAGTG TTTTGATCAT  
 TTTAAATTT TATATGGCGG GTGGTGGGCA ACTCGCTTGC GCGGGCAACT  

40 CGCTTACCGA TTACGTTAGG GCTGATATTT ACGTAAAAAT CGTCAAGGG  
 TGCAAGACCA AAGTAGTAAA ACCCCGGAAG TCAACAGCAT CCAAGCCCAA  
 GTCCTTCACG GAGAAACCCC AGCGTCCACA TCACGAGCGA AGGACCACCT  

45 CTAGGCATCG GACGCACCAT CCAATTAGAA GCAGCAAAGC GAAACAGCCC  
 AAGAAAAGG TCGGCCCCTC GGCCTTTCT GCAACGCTGA TCACGGGCAG  
 CGATCCAACC AACACCCCTCC AGAGTGA CTTGGCGGAAA TTTAAAGGG  

50 TTAATTCCA CTCAACCACA AATCACAGTC GTCCCCGGTA TTGTCCTGCA  
 GAATGCAATT TAAACTCTTC TGCGAATCGC TTGGATTCCC CGCCCCTAGT  
 CGTAGAGCTT AAAGTATGTC CCTTGTCGAT GCGATGTATC ACAACATATA  

55 AATACTAGCA AGGGATGCCA TGCTTGAGG ATAGCAACCG ACAACATCAC  
 ATCAAGCTCT CCCTTCTCTG AACAAATAAAC CCCACAG or a functionally  
 equivalent nucleotide sequence.

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12. A process according to claim 10, wherein the promoter and upstream activating sequences have the following sequence:

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AGATCTGCCCTTATAAATCT CCTAGTCTGA TCGTCGACGC ATTCCGAATA  
 CGAGGCCTGA TTAATGATTA CATAACGCCCTC CGGGTAGTAG ACCGAGCAGC  
 CGAGCCAGTT CAGGCCCTAA AACGCCCTAT ACAATTAAGC AGTTAAAGAA 5  
 GTTACAATCT ACGCTTAAAA AGCTACTTAA AAATCGATCT CGCAGTCCCG  
 ATTGCGCTAT CAAAACCACT TTAAATCAAC TGATTAAGG TGCGAACGA  
 GCTATAAATG ATATAACAAT ATTAAAGCAT TAATTAGAGC AATATCAGGC 10  
 CGCGCACGAA AGGCAACTTA AAAAGCGAAA GCGCTCTACT AAACAGATTA  
 CTTTGAAAAA AGGCACATCA GTATTTAAAG CCCGAATCCT TATTAAGCGC  
 CGAAATCAGG CAGATAAAGC CATAACAGGC AATAGACCTC TACCTATTAA 15  
 ATCGGCTTCT AGGCGCGCTC CATCTAAATG TTCTGGCTGT GGTGTACAGG  
 GGCATAAAAT TACGCCTAC CCGAATCGAT AGAACTACTC ATTTTTATAT  
 AGAAGTCAGA ATTCAATAGTG TTTTGATCAT TTTAAATTT TATATGGCGG 20  
 GTGGTGGGCA ACTCGCTTGC GCGGGCAACT CGCTTACCGA TTACGTTAGG  
 GCTGATATTT ACGTGAAAAT CGTCAAGGGA TGCAAGACCA AAGTAGTAAA  
 ACCCCGGAAG TCAACAGCAT CCAAGCCCAA GTCCTTCACG GAGAAACCCC 25  
 AGCGTCCACA TCACCGAGCGA AGGACCACCT CTAGGCATCG GACGCACCAT  
 CCAATTAGAA GCAGCAAAGC GAAACAGCCC AAGAAAAAGG TCGGCCCCGTC  
 GGCCTTTCT GCAACGCTGA TCACGGGCAG CGATCCAACC AACACCCCTCC 30  
 AGAGTGACTA GGGGCGGAAA TTTAAAGGGG TTAATTCCA CTCAACCACA  
 AATCACAGTC GTCCCCGGTA TTGTCCTGCA GAATGCAATT TAAACTCTTC  
 TGCGAATCGC TTGGATTCCC CGCCCTAGT CGTAGAGCTT AAAGTATGTC 35  
 CCTTGTGAT GCGATGTATC ACAACATATA AATACTAGCA AGGGATGCCA  
 TGCTTGGAGG ATAGCAACCG ACAACATCAC ATCAAGCTCT CCCTTCTCTG  
 40  
 ACAATAAAC CCCACAGAAG GCATTT or a functionally equivalent  
 nucleotide sequence.

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13. A process according to claim 12, wherein the sequence in claim 11 is preceded by the 1.05 kb unsequenced upstream region from position 0 to 1.05 in plasmid pTAKA 17.

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14. A process according to claim 2, wherein the vector system further comprises a preregion providing for secretion of the expressed product into the culture medium.

15. A process according to claim 14, wherein the preregion is derived from a glucoamylase or an amylase gene from an Aspergillus species, an amylase gene from a Bacillus species, a lipase or proteinase gene from Rhizomucor miehei, the gene for the  $\alpha$ -factor from S. cerevisiae or a Humicola lipase gene.

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16. A process according to claim 15, wherein the preregion is derived from the gene for the Humicola lanuginosa lipase, the gene for A. oryzae TAKA amylase, A. niger neutral  $\alpha$ -amylase, A. niger acid-stable  $\alpha$ -amylase, B. licheniformis  $\alpha$ -amylase, the maltogenic amylase from Bacillus NCIB 11837, B. stearothermophilus  $\alpha$ -amylase or B. licheniformis subtilisin.

17. A process according to claim 2, wherein the vector system comprises two vectors, where one contains the selection marker and the other contains DNA-sequences encoding functions facilitating gene expression and a DNA-sequence encoding the desired protein product.

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18. A method for producing of a recombinant Humicola lipase comprising the steps of  
 (a) cultivating an Aspergillus sp. host transformed with a recombinant DNA cloning vector system, said vector system comprising DNA sequences encoding functions facilitating gene expression and a DNA sequence encoding the Humicola sp. lipase, and

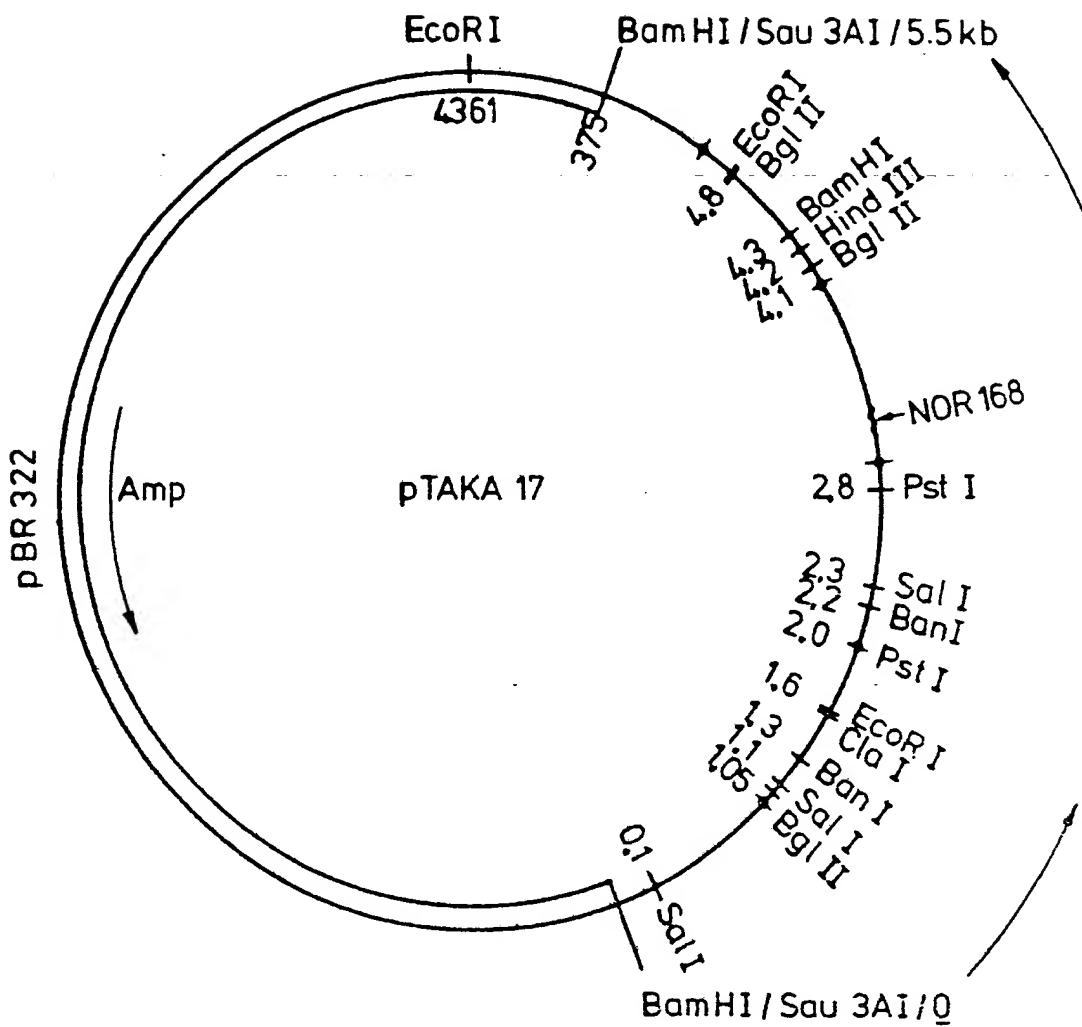
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- (b) recovering said recombinant lipase.
19. A process according to claim 18, wherein the DNA sequences encoding functions facilitating gene expression comprises a promoter, transcription initiation sites, and transcription terminator and polyadenylation functions.
- 5 20. A process according to claim 12, wherein the promoter is the A. oryzae TAKA amylase promoter or functional parts thereof.
21. The method of claim 18 which further comprises recovering said Humicola sp. lipase from the cultivation medium, said recombinant DNA cloning vector system further comprising a preregion providing for secretion operatively linked to the DNA sequence encoding said Humicola sp. lipase.
- 10 22. The method of claim 18, wherein the DNA sequence encodes a Humicola lanuginosa lipase.
23. A process according to claim 18, wherein the Aspergillus sp. host is an Aspergillus oryzae strain.
24. The recombinant Humicola lanuginosa lipase product produced by the process of claim 23, said recombinant lipase product being characterized by greater thermal stability than the native Humicola lanuginosa product and by having a glycosylation different than the glycosylation of the native Humicola lanuginosa lipase.
- 15 25. A recombinant Humicola sp. lipase characterized by having a glycosylation different from the glycosylation of the native Humicola sp. lipase.
26. Recombinant Humicola sp. lipase according to claim 25, wherein the carbohydrate content is from about 5 to about 30% (w/w).
- 20 27. Recombinant Humicola lipase according to claim 26, wherein the carbohydrate content is from about 5 to about 15% (w/w).
28. Recombinant Humicola lipase according to claim 25, wherein the carbohydrate content is from about 6 to about 10% (w/w).
29. Recombinant Humicola lipase according to claim 25, wherein the carbohydrate content is from about 7.5 to about 8.5% (w/w).
- 25 30. Recombinant Humicola lipase according to claim 25, characterized in that it comprises galactose.
31. Recombinant Humicola lipase according to claim 25, characterized in that it comprises N-acetylglucosamine, mannose and galactose.
32. Recombinant Humicola lipase according to claim 30, characterized in that it comprises from about 1 to about 12 mol galactose per mol lipase protein.
33. Recombinant Humicola lipase according to claim 30, characterized in that it comprises from about 1 to about 6 mol galactose per mol lipase protein.
34. Recombinant Humicola lipase according to claim 25, characterized in that it comprises from about 3 to about 20 mol mannose per mol lipase protein.
- 35 35. Recombinant Humicola lipase according to claim 25, characterized in that it comprises from about 3 to about 12 mol mannose per mol lipase protein.
36. Recombinant Humicola lipase according to claim 31, characterized in that it per mol lipase protein comprises about 2 mol N-acetylglucosamine, from about 3 to about 20 mol mannose and from about 1 to about 12 mol galactose.
- 40 37. Recombinant Humicola lipase according to claim 32, characterized in that it per mol lipase protein comprises about 2 mol N-acetylglucosamine, about 3 to about 12 mol mannose and about 1 to about 6 mol galactose.
38. Recombinant Humicola lipase according to claim 32, characterized in that it per mol lipase protein comprises about 2 mol N-acetylglucosamine from about 6 to about 9 mol mannose and from about 2 to about 4 mol galactose.
- 45 39. Recombinant Humicola lipase according to claim 25, characterized in having a residual activity after 2 hours at 60°C of at least 90% at pH from about 6 to about 9.
40. Recombinant Humicola lipase according to claim 25, characterized in having a residual activity after 2 hours at 60°C of at least 80% at pH from about 5.5 to about 9.2.
- 50 41. Recombinant Humicola lipase according to claim 25, characterized in having a residual activity after 2 hours at 55°C of at least 95% at pH from about 6 to about 9.5.
42. Recombinant Humicola lipase according to claim 25, characterized in having a residual activity at 55°C at pH 9 of at about 99%.
- 55 43. Recombinant Humicola lipase according to claim 25, characterized in having a residual activity at 55°C at pH 10 of at least 85%.

FIG. 1  
EP 0305216 A1

4 14 24 34 44 54  
 | | | | | |  
 ATGATGGTCGCGTGGTGGTCTCTATTCTGTACGGCCTTCAGGTCGCGGCACCTGCTTTG  
 Met Met Val Ala Trp Trp Ser Leu Phe Leu Tyr Gly Leu Gln Val Ala Ala Pro Ala Leu  
 64 74 84 94 104 114  
 | | | | | |  
 GCTGCAACGCCCTGCGGACTGGCGATCGCAATCCATTATTCCTTCTCACGGATCGATT  
 Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr Phe Leu Leu Thr Asp Arg Phe  
 mature TAKA-amylase  
 124 134  
 | |  
 GCAAGGACGGATGGGTCGAC  
 Ala Arg Thr Asp Gly Ser

FIG. 2



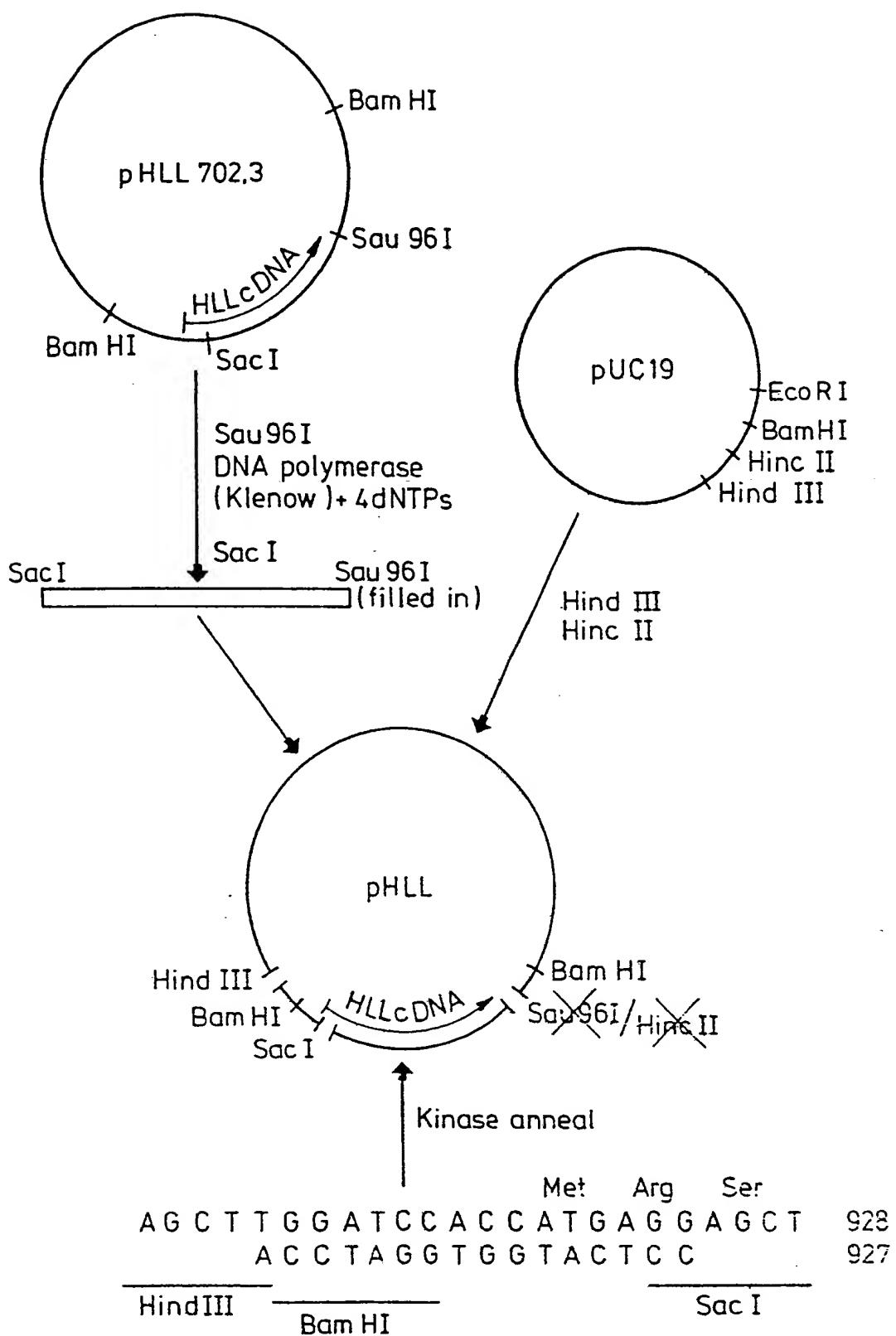


FIG. 4

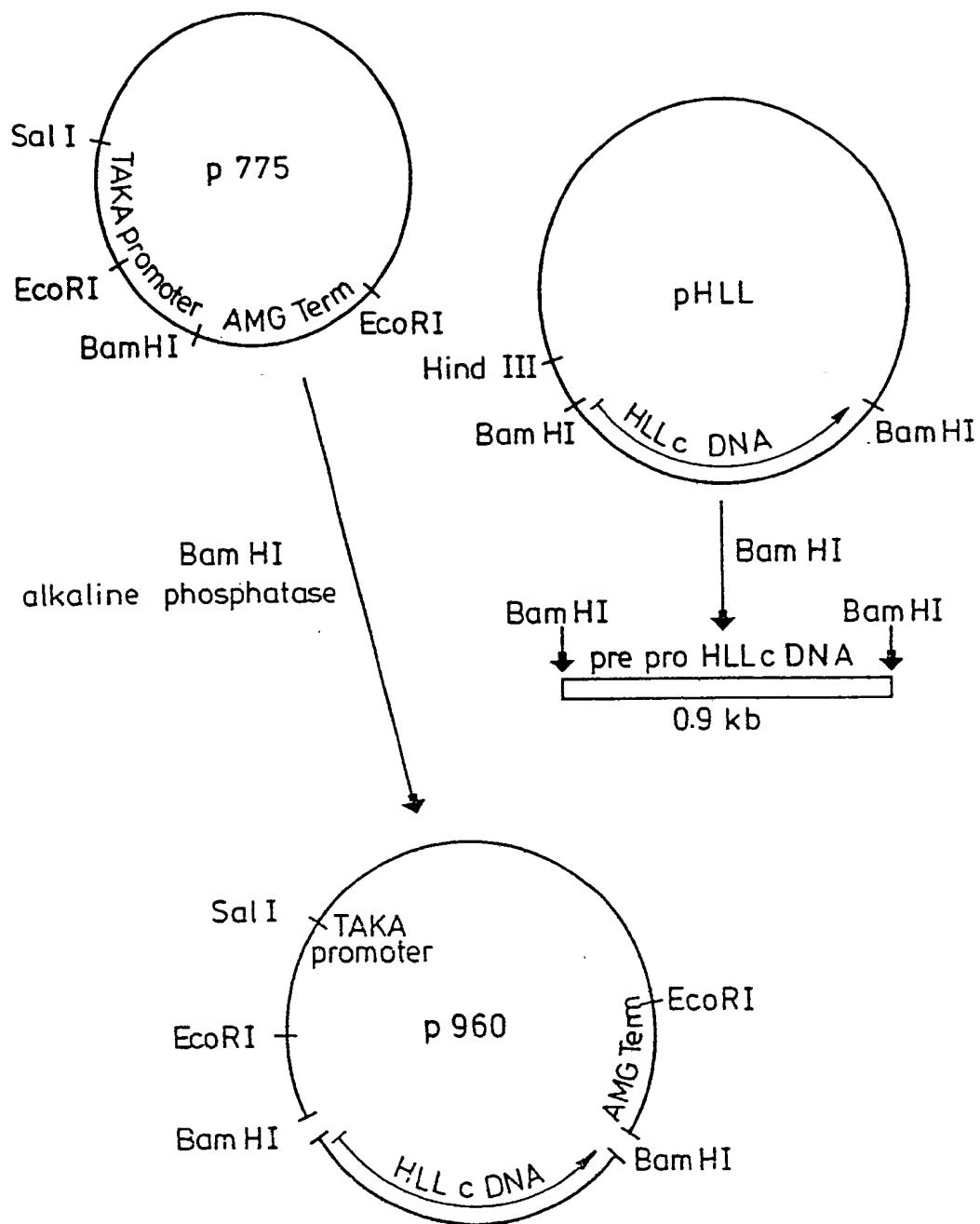


Fig. 5a  
EP 0 305 216 A1

10 20 30 40 50 60 70  
| | | | | | |  
GACAGACAGGTAGAACAAAGACTTGCAACGAATTGATCGATCAGATCGTAGCGACGATATGAGGAGCTCCCT  
METArgSerSerLeu

80 90 100 110 120 130 140  
| | | | | | |  
TGTGCTGTTCTTGTCTCGCGTGGACGGCCTGGCCAGTCCTATTCGTCGAGAGGTCTCGCAGGATCTGTT  
ValLeuPhePheValSerAlaTrpThrAlaLeuAlaSerProIleArgArgGluValSerGlnAspLeuPhe  
↑ Propeptide → AMINO TERM.

150 160 170 180 190 200 210  
| | | | | | |  
TAACCAGTTCAATCTTTGCACAGTATTCTGCAGCCGCATACTGCAGGGAAAAAACATGATGCCAGCTGG  
AsnGlnPheAsnLeuPheAlaGlnTyrSerAlaAlaAlaTyrCysGlyLysAsnAsnAspAlaProAlaGly

220 230 240 250 260 270 280  
| | | | | | |  
TACAAACATTACGTGCACGGAAATGCCTGCCCGAGGTAGAGAAGGCGGATGCAACGTTCTACTCGTT  
ThrAsnIleThrCysThrGlyAsnAlaCysProGluValGluLysAlaAspAlaThrPheLeuTyrSerPhe

290 300 310 320 330 340 350 360  
| | | | | | | |  
TGAAGACTCTGGAGTGGCGATGTCACCGGCTTCCTGCTCTCGACAACACGAACAAATTGATCGCCTCTC  
GluAspSerGlyValGlyAspValThrGlyPheLeuAlaLeuAspAsnThrAsnLysLeuIleValLeuSer

370 380 390 400 410 420 430  
| | | | | | |  
TTTCCGTGGCTCTGTTCCATAGAAGAACTGGATGGAAATTTAACCTCGACTTGAAAGAAATAAATGA  
PheArgGlySerArgSerIleGluGluThrGlySerGlyAsnLeuAsnPheAspLeuLysGluIleAsnAsp

440 450 460 470 480 490 500  
| | | | | | |  
CATTTGCTCCGGCTGCAGGGGACATGACGGCTTCACCTCGTCCGGAGGTCTGTAGCCGATACGTTAAGGCA  
IleCysSerGlyCysArgGlyHisAspGlyPheThrSerSerTrpArgSerValAlaAspThrLeuArgGln

510 520 530 540 550 560 570  
| | | | | | |  
GAAGGTGGAGGATGCTGTGAGGGAGCATCCCGACTATCGCGTGGTGTACCGGACATAGCTTGGGTGGTGC  
LysValGluAspAlaValArgGluHisProAspTyrArgValValPheThrGlyHisSerLeuGlyGlyAla

Fig. 5b

EP 0 305 216 A1

580        590        600        610        620        630        640  
|            |            |            |            |            |            |  
ATTGGCAACTGTTGCCGGAGCAGACCTGCGTGGAAATGGGTATGATATCGACGTGTTTCATATGGCGCCCC  
LeuAlaThrValAlaGlyAlaAspLeuArgGlyAsnGlyTyrAspIleAspValPheSerTyrGlyAlaPro

650        660        670        680        690        700        710        720  
|            |            |            |            |            |            |            |  
CCGAGTCGGAAACAGGGCTTTGCAGAATTCTGACCGTACAGACCGGGGAACACTCTACCGCATTACCCA  
ArgValGlyAsnArgAlaPheAlaGluPheLeuThrValGlnThrGlyGlyThrLeuTyrArgIleThrHis

730        740        750        760        770        780        790  
|            |            |            |            |            |            |  
CACCAATGATATTGTCCCTAGACTCCCGCCGCGAATTGGTTACAGCCATTCTAGCCCAGAGTACTGGAT  
ThrAsnAspIleValProArgLeuProProArgGluPheGlyTyrSerHisSerSerProGluTyrTrpIle

800        810        820        830        840        850        860  
|            |            |            |            |            |            |  
CAAATCTGGAACCCTTGTCCCCGTACCCGAAACGATATCGTGAAGATAGAAGGCATCGATGCCACCGCGG  
LysSerGlyThrLeuValProValThrArgAsnAspIleValLysIleGluGlyIleAspAlaThrGlyGly

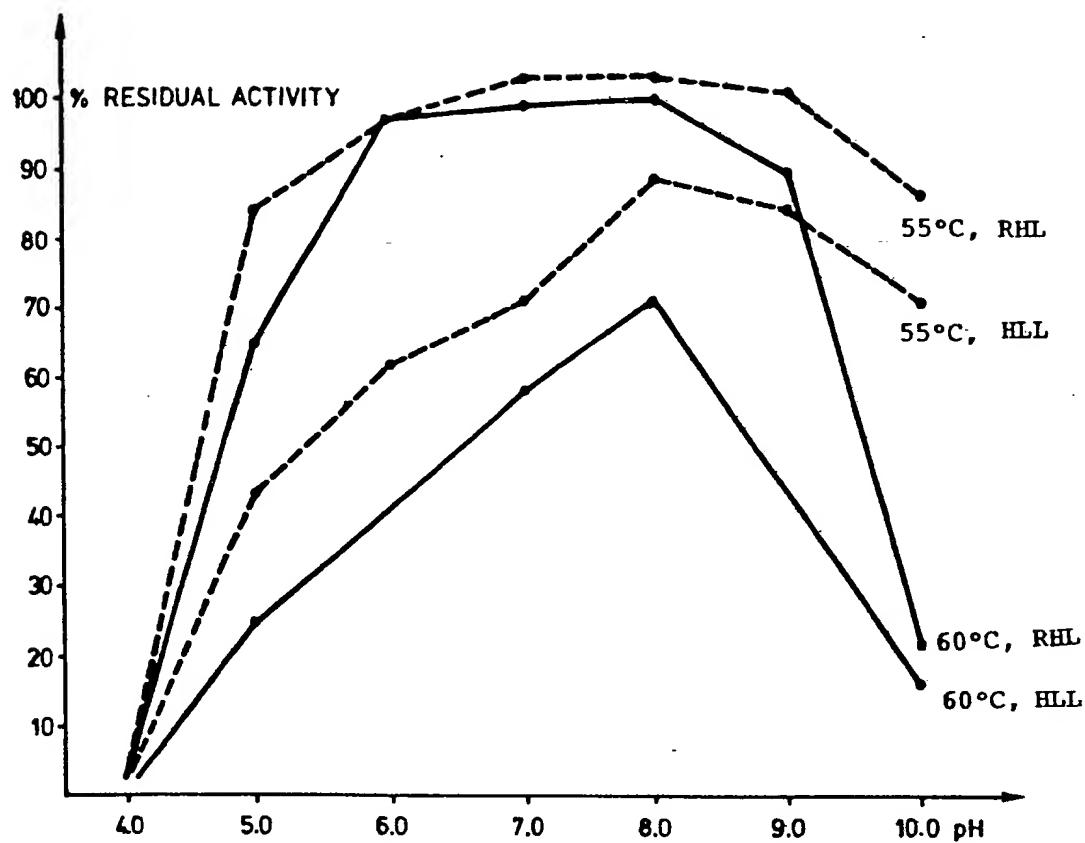
870        880        890        900        910        920        930  
|            |            |            |            |            |            |  
CAATAACCAGCCTAACATTCCGGATATCCCTGCGCACCTATGGTACTTCGGGTTAATTGGGACATGTCTTTA  
AsnAsnGlnProAsnIleProAspIleProAlaHisLeuTrpTyrPheGlyLeuIleGlyThrCysLeu  
STOP

940        950        960        970        980        990        1000  
|            |            |            |            |            |            |  
GTGCGCGCGCGGGCTGGGTCCGCAATGACAGTTGTATAAAGTTGAGGTTAGGCAGGATCATGATGTTCGT

1010        1020        1030        1040        1050        1060        1070        1080  
|            |            |            |            |            |            |  
CACTTGGGGTCATTTGACGGTCAAATCAAGATGACACTCTCCAAGCATTGATGAGTTGAATCAAATGGATC

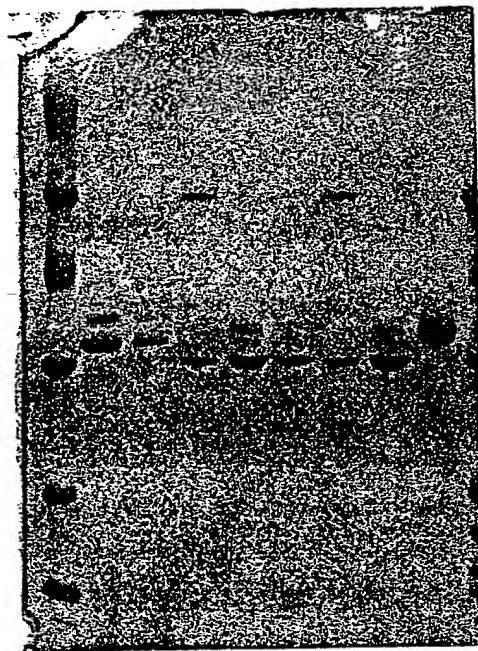
1090        1100        1110  
|            |            |  
AGTGGTACATAACGCAATGCCGTATTATTTGA polyA

FIG. 6



EP 0 305 216 A1

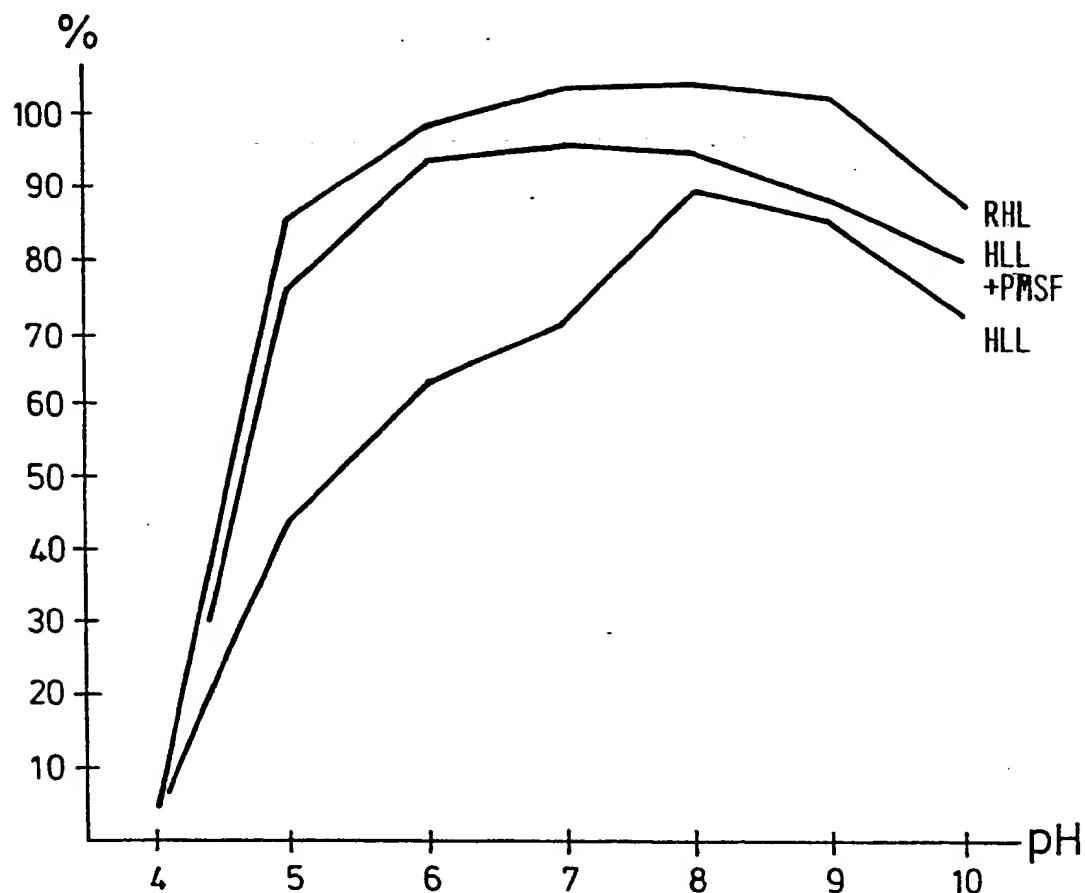
Fig. 7



1 2 3 4 5 6 7 8 9

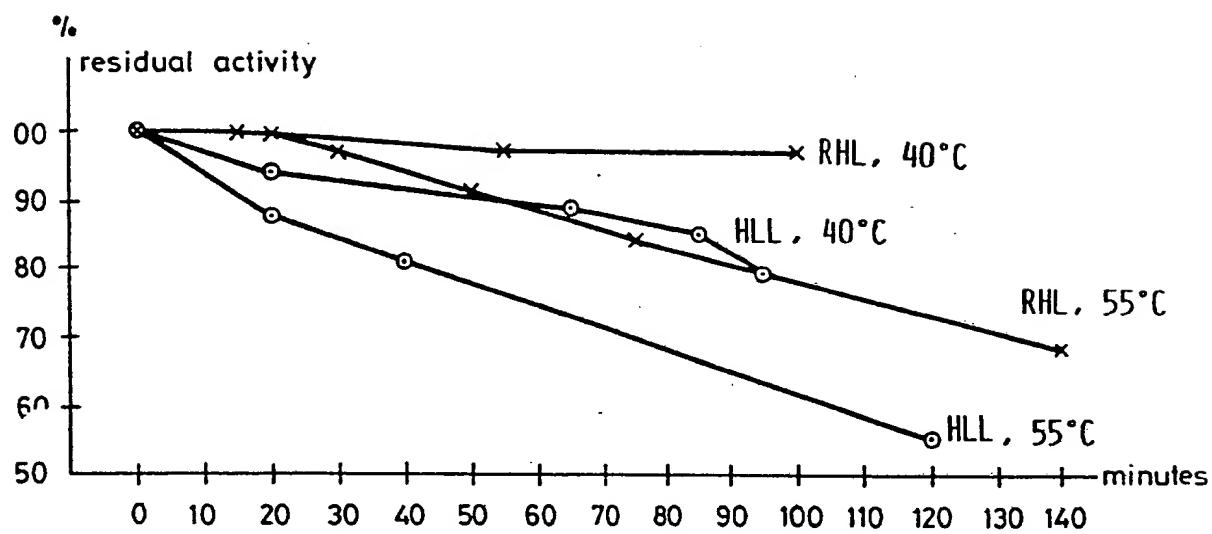
FIG. 8

Residual activity



pH stability profile  
( 2 hours at 55° C )

FIG. 9





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EP 88 30 7980

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y, D P	EP-A-0 258 068 (NOVO INDUSTRI A/S) * Whole document * ---	1-43	C 12 N 15/00 C 12 N 9/20
Y	BIOLOGICAL ABSTRACTS, vol. 56, no. 12, no. 68797, Philadelphia, PA, US; W. H. LIU et al.: "Purification and general properties of the lipase of thermophilic fungus <i>Humicola lanuginosa</i> S-38", & AGRIC. BIOL. CHEM. 37(1): 157-163, 1973. * Whole abstract * ---	1-43	
Y	CHEMICAL ABSTRACTS, vol. 83, 1975, page 401, abstract no. 112391y, Columbus, Ohio, US; & JP-A-75 63 188 (YAMASA SHOYU CO., LTD) 29-05-1975 * Whole abstract * ---	1-43	
Y, D P	EP-A-0 238 023 (NOVO INDUSTRI A/S) * Examples 8,9,14; figures 13a,b; claims * ---	1-43	
Y	EP-A-0 215 594 (GENENCOR INC.) * Whole document * ---	1-9,14- 16,18- 19,21- 23	TECHNICAL FIELDS SEARCHED (Int. Cl.4)  C 12 N
Y	EP-A-0 184 438 (ALLELIX INC.) * Whole document * ---	1-9,14- 16,18- 19,21- 23	
		-/-	
<p>The present search report has been drawn up for all claims</p>			
Place of search  THE HAGUE	Date of completion of the search  22-11-1988	Examiner  CASTRO Y GONZALEZ R.F.	
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- &amp; : member of the same patent family, corresponding document</p>	
<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>			



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Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Y	THE EMBO JOURNAL, vol. 4, no. 2, 1985, pages 475-479, IRL Press Ltd, Oxford, England; J.M KELLY et al.: "Transformation of <i>Aspergillus niger</i> by the <i>amdS</i> gene of <i>Aspergillus nidulans</i> " * Whole article * ---	1-9, 14-19, 21-23	
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 141, no. 1, 26th November 1986, pages 185-190, Academic Press Inc.; W. KUGIMIYA et al.: "Molecular cloning and nucleotide sequence of the lipase gene from <i>Pseudomonas fragi</i> " * Whole article * -----	1-43	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
<p>The present search report has been drawn up for all claims</p>			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	22-11-1988	CASTRO Y GONZALEZ R.F.	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			